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Suppressive Oligonucleotides Protect Against Collagen-Induced Arthritis in Mice

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Objective. **To examine whether systemic administration of oligonucleotides (ODNs), known to inhibit the production of proinflammatory cytokines, alters host susceptibility to collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis (RA).**

Methods. **CIA was induced by injecting DBA/1 mice with type II collagen (CII) in Freund's complete adjuvant, followed 3 weeks later by CII in Freund's incomplete adjuvant. The effect of suppressive ODNs on the incidence and severity of disease was monitored, as were immune correlates of CIA.**

Results. **Suppressive ODNs administered during the inductive phase of CIA significantly reduced the incidence and severity of arthritis. Treatment with suppressive ODNs significantly decreased serum titers of pathogenic IgG anti-CII autoantibodies and interferon-γ production by collagen-reactive T cells.**

Conclusion. **Suppressive ODNs may be of therapeutic value in the treatment of RA, and potentially other autoimmune diseases.**

Rheumatoid arthritis (RA) is the most common rheumatic disease. It is characterized by progressive destruction, deformity, and disability of the joints, and patients are at risk of premature death (1). Although the etiology of RA remains unknown, bacterial infection may be an important disease trigger (2).

Collagen-induced arthritis (CIA) is a well-established murine model of RA that has helped in the examination of potential treatments and in the clarification of the pathogenesis of disease (3). CIA is induced by repeatedly injecting DBA/1 mice with type II collagen (CII; an autoantigen present in cartilage) emulsified in Freund's complete adjuvant (CFA). The inflammatory response elicited by CII is promoted by bacterial products present in CFA, modeling the contribution of bacterial products to the development of RA.

Recent studies demonstrate that CpG motifs present in bacterial DNA can contribute to the development of arthritis (4,5). This effect is inhibited by suppressive oligonucleotides (ODNs) capable of blocking CpG-induced production of proinflammatory cytokines and chemokines (5,6). Suppressive ODNs contain "poly-G" motifs similar to those present at high frequency in mammalian, but not bacterial, genomes, where they may function to suppress inflammatory responses injurious to host tissue (4,7). Although suppressive ODNs can inhibit the immune activation and arthritis induced by CpG motifs (4,8,9), their ability to reduce arthritis in an unrelated disease model is uncertain.

This study examined whether suppressive ODNs can down-regulate the autoimmune response against CII. The results indicate that administering suppressive ODNs during the inductive phase of CIA reduces the incidence and attenuates the severity of arthritis in DBA/1 mice. These beneficial effects are associated with the inhibition of antigen-specific cellular and humoral immune responses.

MATERIALS AND METHODS

Disease model. Male DBA/1 LacJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen–free conditions. All studies were approved by the Center for Biologics Evaluation and Research (CBER) Animal Care and Use Committee.

Mice (8–10 weeks old) were injected intradermally at the base of the tail with 100 μg bovine CII (Elastin Products, Owensville, MO) emulsified in CFA and 21 days later with CII in Freund's incomplete adjuvant (IFA). Mice were examined daily for evidence of joint swelling/inflammation. The "arthritis clinical score" of each paw was based on the following grading

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EXHIBIT A

scale: 0 = normal, 1 = mild swelling of a single joint or focal redness, 2 = moderate swelling of joints, and 3 = severe swelling or joint rigidity and bony deformity.

ODNs. Phosphorothioate ODNs were synthesized at the CBER Core Facility. Sequences of the ODNs used in experiments were as follows: for suppressive ODN A151, TTAGGGTTAGGGTTAGGGTTAGGG and for control ODN 1612, GCTAGATGTTAGCGT. There was no detectable protein or endotoxin contamination of these ODNs.

Mice were injected intraperitoneally with 300 μg ODNs dissolved in 300 μl phosphate buffered saline (PBS). Groups of mice received a single treatment on day −3 or on day 3, 2 treatments on days −3 and 3, days 0 and 21, or days −3 and 18; or repeated treatments on days −3, 0, 3, 7, 10, 14, 18, and 21 relative to the administration of CII in CFA.

Serum anti-CII antibody levels. Serum was collected on day 35 and stored frozen until used. Immulon IB microtiter plates (Thermo Electron Corporation, Waltham, MA) were coated with 2 μg/ml CII in PBS, blocked with 1% bovine serum albumin (BSA) in PBS, and washed. Diluted serum was added for 2 hours, plates were washed, and bound antibodies were detected by the addition of alkaline phosphatase–conjugated goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL). Antibody concentration was determined by comparison with a standard curve generated using high-titered antiserum.

Cell culture and interferon-γ (IFNγ) assay. Spleen cells were isolated on day 22 or 35. Cells (5×10^5/well) were cultured with 50 μg/ml CII for 72 hours. IFNγ levels in culture supernatants were detected by enzyme-linked immunosorbent assay. Briefly, 96-well plates were coated with anti-IFNγ antibody (BioSource International, Camarillo, CA) and blocked with PBS–1% BSA. Bound cytokine was detected by the addition of biotin-labeled anti-IFNγ antibody, followed by phosphatase-conjugated avidin.

Statistical analysis. A one-way analysis of variance was used to study the data in Table 1 and Figure 1. The Mann-Whitney U test was used to analyze serum anti-CII antibody levels, while Student's *t*-test was used to analyze the IFNγ levels (Figure 2). Differences between experimental groups were analyzed by Mann-Whitney U test or Student's *t*-test.

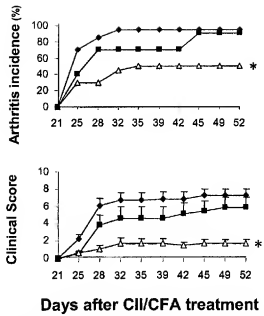

Figure 1. Effect of suppressive oligonucleotide (ODN) treatment on the incidence and severity of arthritis. Arthritis was induced by injecting DBA/1 mice with type II collagen (CII) in Freund's complete adjuvant (CFA), followed 3 weeks later by CII in Freund's incomplete adjuvant (all injections were intradermal). Suppressive ODN A151 (△), control ODNs (■), or phosphate buffered saline (PBS; ◆) was administered intraperitoneally twice weekly during the induction phase of collagen-induced arthritis (CIA) (see Materials and Methods for details). The incidence (**top**) and severity (**bottom**) of CIA were evaluated. Values are the mean and SEM of 2 identical experiments involving 10 mice per group, and thus provide results from a total of 20 mice per group. * = $P < 0.05$ versus control ODN– or PBS-treated mice.

RESULTS

Suppressive ODNs protect DBA/1 mice from CIA. CIA was elicited by injecting DBA/1 mice with CII in CFA on day 0 and in IFA 3 weeks later (3). As shown in Figure 1, this procedure induced arthritis of the small joints in 95% of DBA/1 mice. Joint swelling developed several days after the second CII injection and persisted for many weeks.

To evaluate whether suppressive ODNs could reduce arthritis in this model, ODN A151 was administered once, twice, or multiple times to CII-injected mice. A single ODN A151 treatment (before or after delivery of CII in CFA) had no significant impact on the incidence or severity of arthritis (Table 1). Two treatments with ODN A151 reduced disease severity ($P < 0.05$) but had no effect on the incidence of arthritis (Table 1). By comparison, when ODN A151 was delivered twice per week starting 3 days prior to CII injection, the incidence of CIA was reduced by half ($P < 0.05$) (Figure 1, top), and disease severity was reduced nearly 5-fold as compared with a PBS-treated group ($P < 0.05$) (Figure 1, bottom). This beneficial effect was unequivocally attributed to the administration of suppressive ODNs, since control ODNs had no effect on disease progression (Table 1).

Histologic analysis of the joints from mice with CIA showed severe lymphocytic infiltration, erosion of bone and cartilage, active proliferation of synovial lining cells, and disruption of the joint (data not shown). By comparison, joints from mice treated with ODN A151

Table 1. Effect of suppressive oligonucleotides (ODNs) on the development of collagen-induced arthritis (CIA)*

Treatment	Incidence	Clinical score
Suppressive ODNs		
One injection	18/20	5.3 ± 0.6
Two injections	25/30	3.9 ± 0.5†
Multiple injections	10/20	1.5 ± 0.4‡
Control ODNs		
Multiple injections	9/10	5.4 ± 1.2
PBS	19/20	6.9 ± 0.8

* DBA/1 mice were injected intradermally with 100 μg of type II collagen (CII) in Freund's complete adjuvant, followed 3 weeks later by CII in Freund's incomplete adjuvant, to induce CIA. Mice were injected with 300 μg of suppressive ODN A151, control ODN 1612, or phosphate buffered saline (PBS) at the times described in Materials and Methods. Incidence reflects the number of mice with arthritis/total number of mice per group. Clinical score represents the mean ± SEM combined incidence and clinical score of mice 42 days after initial CII administration from 3 independent experiments.
† $P < 0.05$ compared with the PBS control group.
‡ $P < 0.05$ compared with the control ODN group.

during the induction of CIA showed only mild cellular infiltration without disruption of the joint architecture.

Inhibition of anti-CII antibody production by suppressive ODNs. The pathogenesis of CIA involves both humoral and cell-mediated immune responses directed against the CII protein (10). Serum titers of IgG anti-CII autoantibodies were measured at the peak antibody response on day 35. Treatment with ODN A151 significantly reduced serum levels of IgG anti-CII antibodies as compared with PBS- or control ODN–treated mice ($P < 0.05$) (Figure 2, top). Of particular relevance, suppressive ODN treatment reduced the concentration of IgG2a and IgG2b anti-CII antibodies (previously shown to contribute to disease pathogenesis) by >40% and >70%, respectively ($P < 0.05$ in each case).

Inhibition of antigen-specific IFNγ production. The production of Th1 cytokines, such as IFNγ, plays an important role in the development of CIA (11). Consistent with previous studies, large amounts of IFNγ were produced by spleen cells from CIA mice stimulated in vitro with CII antigen (Figure 2, bottom). The same level of IFNγ production was elicited when splenocytes from CIA mice treated with control ODNs were stimulated. In contrast, spleen cells treated with ODN A151 during the inductive phase of CIA failed to produce IFNγ when cultured with CII in vitro ($P < 0.05$) (Figure 2).

DISCUSSION

This study examined the effect of administering suppressive ODNs during the inductive phase of CIA on immunologic and clinical parameters of disease in DBA/1 mice. Suppressive ODNs were initially identified by their ability to neutralize the immune activation elicited by CpG DNA (4). In an experimental model of CpG-induced reactive arthritis, both intraarticular and systemic administration of suppressive ODNs prevented the development of joint inflammation (8,9). Yet, recent studies indicate that suppressive ODNs have more general effects on the immune system, blocking nitric oxide production by macrophages, preventing lipopolysaccharide-induced activation of dendritic cells, and down-modulating Th1 cytokine production (6,12,13). This raised the possibility that suppressive ODNs might be of broader use in the prevention or treatment of inflammatory and/or autoimmune disorders.

CIA is an experimental model of RA that closely mimics human RA. Both diseases are characterized by

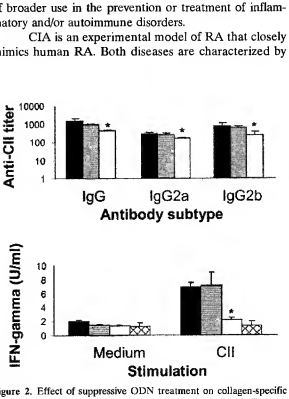

Figure 2. Effect of suppressive ODN treatment on collagen-specific immune responses. DBA/1 mice were treated as described in Figure 1 to induce CIA, and studied on day 35. **Top,** IgG anti-CII antibody titers were measured in the serum of mice treated with PBS (solid bar), control ODNs (shaded bar), or suppressive ODNs (open bar). Values are the geometric mean and SEM titers. **Bottom,** Spleen cells were isolated from CIA mice treated with PBS, control ODNs, or suppressive ODNs, or from naive mice (hatched bar). These cells were cultured in medium alone or were stimulated with 50 μg/ml CII. Interferon-γ (IFNγ) levels in culture supernatants 2 days later were measured by enzyme-linked immunosorbent assay. Values are the mean and SEM of 2 independent experiments (n = 6). * = $P < 0.05$ versus PBS- or control ODN–treated mice. See Figure 1 for other definitions.

humoral and cell-mediated responses against the CII autoantigen. IgG2a and IgG2b anti-CII autoantibodies are particularly pathogenic (3), although disease severity is influenced by antigen-specific Th1 and proinflammatory cytokine production (14). While interventions beneficial to the prevention and treatment of RA have been developed, agents capable of blocking the immune stimulation that contributes to the induction and/or exacerbation of inflammation could complement existing therapies.

Current results indicate that suppressive ODNs administered during the inductive phase of disease can inhibit the inflammatory immune response induced by CII. This finding confirms the ability of suppressive ODNs to prevent autoimmune processes that are not induced by CpG DNA, as first documented in a murine model of experimental autoimmune encephalomyelitis (EAE) (13). Whereas a single dose of suppressive ODNs failed to protect mice from CIA, 2 treatments reduced disease severity and twice-weekly treatments reduced both the frequency and severity of disease (Table 1). Clinical benefits were confirmed by histologic analysis of the joints and by analysis of joint levels of inflammatory cytokines such as interleukin-1β (data not shown). These effects were unequivocally ascribed to the use of suppressive ODNs, since control ODNs had no effect on disease.

The mechanism by which suppressive ODNs reduce the severity of CIA was explored. Previous studies established that suppressive ODNs inhibit the production of various Th1 and proinflammatory cytokines (4,7). Results of the current study demonstrate that in vivo treatment of mice with ODN A151 significantly reduces CII-dependent IFNγ secretion (Figure 2). In addition, treatment with ODN 151 reduces serum anti-CII antibody titers by >40%, including autoantibodies of the most pathogenic IgG2a and IgG2b subtypes (Figure 2). As noted above, CII-reactive IFNγ-producing T cells and anti-CII autoantibodies can synergistically enhance the development of CIA. Thus, the ability of suppressive ODNs to inhibit the production of both pathogenic autoantibodies and autoantigen-specific IFNγ provides a basis for the observed protection from disease.

The current results in combination with evidence that suppressive ODNs prevent the development of CpG-induced inflammatory arthritis and myelin-induced EAE (9,13) suggest that these agents may be broadly useful in the prevention or treatment of autoimmune disease. Consistent with such a conclusion, preliminary studies in our laboratory suggest that suppressive ODNs may reduce the frequency and severity of disease in lupus-prone mice. Such findings support continued exploration of the therapeutic potential of suppressive ODNs, and efforts to better understand the molecular mechanism by which they inhibit inflammatory reactions.

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